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Dystrophin is transcribed in brain from a distant upstream promoter

(muscular dystrophy/mental retardation)

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ABSTRACT **Dystrophin, the protein product of the Duchenne muscular dystrophy gene, is expressed in brain as well as muscle. The role of dystrophin in the brain is not clear, though one-third of Duchenne muscular dystrophy patients exhibit some degree of mental retardation. We have isolated the genomic region encoding the alternative 5′ terminus of dystrophin used in the brain. Primer extension and polymerase chain reaction assays on RNA demonstrate that this region contains an alternative promoter for dystrophin used in the brain. Physical mapping of this region indicates that this brain promoter is located >90 kilobases 5′ to the promoter used in muscle and 400 kilobases from exon 2 to which it is spliced. The large physical distance between the promoters, taken together with their known tissue selectivities, suggests that in certain patients a deletion of either dystrophin promoter might give rise to reduced dystrophin expression selective to brain or muscle. We have identified one such individual with specific deletion of the dystrophin muscle promoter, giving rise to Becker muscular dystrophy, and we predict that specific loss of the brain promoter may be one cause of X chromosome-linked mental retardation.**

Duchenne muscular dystrophy (DMD) is a relatively common and uniformly lethal inherited disorder. The disease primarily affects muscle, with symptoms of proximal muscle weakness first appearing by age 5 and progressing to respiratory or cardiac failure and death by the third decade. Much insight into the pathophysiology of Duchenne muscle has come from analysis of dystrophin, the protein product of the DMD gene (1). Dystrophin, a large (427 kDa) protein with amino acid sequence homology to the spectrin family of membrane cytoskeletal proteins (2–4), is most abundant in skeletal and cardiac muscle, where it has been localized to the inner face of the plasma membrane (5–8). Dystrophin is also found throughout the body in smooth muscle (9). DMD results from severely reduced amounts of dystrophin, whereas the milder Becker muscular dystrophy results primarily from mutations that generate dystrophin of abnormal molecular weight or quantity (10, 11). It is hypothesized that dystrophin might serve to stabilize the plasma membrane during muscle fiber contraction and that absence of dystrophin leads to membrane disruption and muscle fiber necrosis (6, 12). The only nonmuscle tissue that expresses significant levels of dystrophin is the brain (1, 9, 13, 14).

The presence of dystrophin in the brain has revived interest in the possible central nervous system manifestations of DMD. Studies of Duchenne patients have revealed a moderate depression of IQ scores, with up to one-third of Duchenne patients being classified as mentally retarded (reviewed in ref. 15). Dystrophin mRNA and protein are present in brain at about 1/10th the low level found in skeletal muscle (1, 9, 13, 14), making analysis of dystrophin in the brain difficult. Nevertheless, it is known that dystrophin mRNA in the brain contains different sequences at the 5′ terminus from that found in muscle, suggesting that dystrophin is transcribed from an alternate promoter or is alternatively spliced in the brain (16, 17). Low levels of the brain-type 5′ terminus have been found in muscle, and low levels of the muscle transcript have been observed in the brain (16, 18). Therefore, the alternative 5′ terminal sequences appear to show tissue selectivity rather than strict specificities.

To study the expression of dystrophin in the brain, we isolated genomic sequences encoding the 5′ region of dystrophin found in brain transcripts†. We demonstrate that this genomic region contains an alternate promoter for dystrophin in brain, which is located >90 kilobases (kb) upstream of the DXS142 region containing the major muscle promoter (19). The finding that the DMD gene contains dual promoters, which are widely spaced, suggests that deletion mutations may disrupt expression of one tissue-selective promoter without affecting expression in the other tissue, thus giving rise to novel phenotypes. We have identified one such example in a patient with Becker muscular dystrophy caused by specific deletion of the muscle promoter.

EXPERIMENTAL PROCEDURES

Genomic Cloning and Sequence Analysis. A normal human female genomic library in EMBL3 was screened using a ^{32}P-labeled-polymerase chain reaction (PCR) probe prepared as described (20). Phage growth, library screening, phage DNA isolation, and subcloning were by routine procedures (21). DNA sequencing was performed using the Sequenase kit version 2.0 (United States Biochemical).

RNA Analysis. PCR was performed directly on RNA as described (22). The antisense exon 3 primer was 5′-TAGGTCACTGAAGAGGTTCTCAATATGCTG-3′. Primer extensions were performed as described (21) by using the primer 5′-CCGGAAGCTTGATTCTGTCATCTTCCTGAAAGCA-3′, which was 5′ labeled with [γ-^{32}P]ATP (NEN) and T4 polynucleotide kinase (United States Biochemical). COS7 cells were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum and electroporated at 280 V and 960 μF by using a Bio-Rad electroporator. The cells were harvested 42 hr posttransfection, and poly(A)$^+$ RNA was isolated by using the Fast Track kit (Invitrogen, San Diego).

Reporter Gene Construction. A *Hin*dIII site was introduced into the brain promoter region by PCR amplification of a 6.2-kb *Hin*dIII subclone of the brain promoter region in Bluescript SK II+ by using the primer 5′-CCGGAAGCTTGATTCTGTCATCTTCCTGAAAGCA-3′ and a T7 primer

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Abbreviations: DMD, Duchenne muscular dystrophy; PCR, polymerase chain reaction.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M59228).

complementary to the vector. The resulting product, containing 3 kb of 5′ flanking region as a *Hin*dIII fragment, was subcloned into a polylinker-containing derivative of pSV0 A/L Δ 5′ (23). To allow expression in COS cells, a fragment containing the simian virus 40 enhancer was prepared by PCR of pSV2CAT (24) and subcloned into the *Xho* I site of the polylinker. The resulting construct, pB-Luc/E, contained 3 kb of 5′ flanking sequence from the upstream promoter of dystrophin directing expression of the luciferase reporter gene, with the simian virus 40 enhancer located upstream of the dystrophin sequences. These plasmids and details of their construction are available upon request.

Pulsed-Field Gel Analysis. Preparation of DNA in agarose blocks and restriction enzyme digestions were performed as described (25). Digested DNA was separated in 1% agarose gels in 45 mM Tris borate/1 mM EDTA buffer by using an LKB Pulsaphor unit with either the double inhomogenous field electrodes at 330 V with alternating switching times of 15, 30, and 60 sec at 9°C for 33 hr (Fig. 4 *A* and *B*) or with the LKB hexagonal electrode array at 170 V, 10-sec switching, 11°C for 22 hr (Fig. 4 *C* and *D*), both as described (25).

Patient Analysis. PCR analysis was performed on 250 ng of genomic DNA as described (26). The brain promoter region was amplified by using the primers 5′-GAAGATC-TATATTTTACAACGCAGAAATGTGG-3′ and 5′-CTTC-CATGCCAGCTGTTTTTCCTGTCACTC-3′. The muscle promoter region was amplified by using the primers 5′-GAAGATCTAGACAGTGGATACATAACAAATG-CATG-3′ and 5′-TTCTCCGAAGGTAATTGCCTCCCA-GATCTGAGTCC-3′. Random-primed probes were prepared by using a DNA labeling kit (Boehringer Mannheim), and filters were hybridized and washed as described (27). Western blot analysis was performed as described (10).

RESULTS

Dystrophin Is Transcribed in Brain From an Alternate Promoter. Previous reports have demonstrated that dystrophin mRNA from brain tissue contained different 5′-terminal sequences from that found in muscle (16, 17). To determine whether this difference was the result of alternative splicing or differential promoter usage, the genomic region encoding these alternative sequences was isolated. Four independent λ phage encompassing 28 kb of genomic sequence were obtained by screening a human genomic library with a probe derived from the 5′ terminus of a dystrophin cDNA from human brain. The DNA sequence immediately 5′ to this cDNA was determined and is shown in Fig. 1. To map the 5′ terminus of this exon, a series of PCR primers was designed successively further upstream from the known cDNA sequence (P1–P6, Fig. 1) and used in combination with an exon 3 antisense primer in PCR reactions on human brain RNA. As shown in Fig. 2*A*, only the most proximal primer, P6, was able to amplify the proper product from human brain poly(A)$^+$ RNA. As a control, the same set of primers was also used on genomic DNA in combination with an exon 1 antisense primer, and all were able to amplify the proper sized products (Fig. 2*B*). Thus, all of these primers are capable of efficient amplification of this template. This suggests that the endogenous dystrophin transcript in the brain initiates or is spliced within the primer P5 site or between the primer P5 and primer P6 sites.

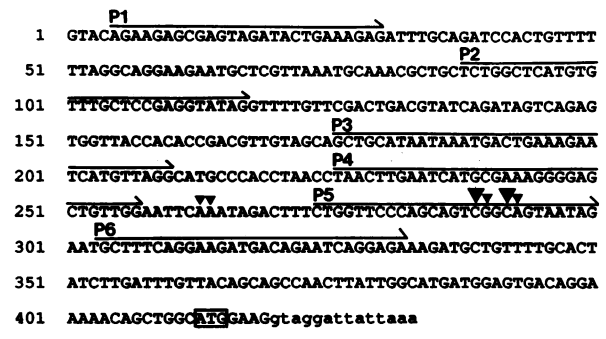

FIG. 1. DNA sequence of the dystrophin alternative exon and flanking sequences. The 5′ flanking and exon sequences are in uppercase letters; the intron sequence is in lowercase letters. The translation initiation site is boxed. The locations of major and minor transcription initiation sites (as determined in Fig. 3) are indicated by large and small triangles, respectively. The 5′ terminus of the previously described human cDNA from this region (16) is at base 303. Primers used in Fig. 2 are designated P1–P6.

To map the 5′ terminus of the dystrophin transcript within this region, primer extension was performed on human brain RNA and on RNA from cells transfected with this putative promoter region directing expression of a luciferase reporter gene. The level of expression of the endogenous gene in brain was below that detectable by primer extension (Fig. 3, lane 3). However, in RNA from transfected cells, two major and several minor primer extension products are observed (Fig. 3, lane 1), which are not observed in RNA from untransfected control cells (Fig. 3, lane 2). The location of these two major transcription initiation sites in the transfected cells is just upstream from the primer P6 site (Fig. 1). This result is consistent with the observation that brain RNA was amplified with primer P6 but not with primers P1–P5 (Fig. 2*A*), although transcription initiated at the minor upstream site should yield some degree of amplification with primer P5 as well. Thus, our data suggest that the majority of transcription from this region in human brain initiates just upstream of the primer P6 site. The sequence of this region does not contain any strong TATA box motifs (28, 29), although there is an A+T-rich region centered 26 bases upstream of the major start sites (Fig. 1).

The Dystrophin Brain Promoter Is Located a Minimum of 90 kb Upstream of the Muscle Promoter and ≈400 kb Upstream of Exon 2. To determine the genomic order and locations of the alternative dystrophin promoters, pulsed-field gel electrophoresis was performed. The muscle promoter has been previously mapped to *Sfi* I fragments of ≈700 and 840 kb, depending upon the DNA methylation state of the cell line used (refs. 30–33, Fig. 4*B*). Pulsed-field gel electrophoresis analysis using probes from the brain promoter region revealed an *Sfi* I fragment of 140 kb (Fig. 4*A*), suggesting that

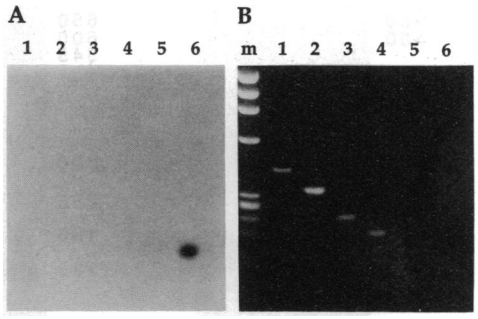

FIG. 2. PCR analysis of endogenous brain transcripts. (*A*) Southern blot of PCR products from human fetal brain RNA using a series of primers P1–P6 (lanes 1–6) in the sense orientation and an antisense primer complementary to sequences within the third exon. The blot was probed with an internal oligonucleotide from the first exon. Longer exposures of this blot did not show any amplification from primers P1–P5. (*B*) The same series of primers P1–P6 was used in conjunction with a primer complementary to exon 1 sequences to amplify products from genomic DNA. In contrast to PCR analysis on RNA, all of these primers were able to amplify this template from genomic DNA. Lane M contains *Hae* III-digested φX174 markers.

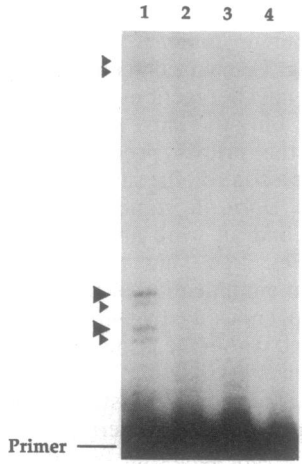

FIG. 3. Primer extension of transfected and endogenous dystrophin genes. A 5'-labeled oligonucleotide complementary to the alternative first exon of dystrophin was hybridized to 5 μg of poly(A)$^+$ RNA from various sources and extended at 42°C by using avian reverse transcriptase. The RNAs used were from COS cells transfected with a dystrophin upstream brain promoter–luciferase reporter gene construct (pB-Luc/E) (lane 1), untransfected Cos cells (lane 2), human fetal brain (lane 3), and no RNA (lane 4). The locations of the extension stop points (triangles) were determined by parallel lanes containing dideoxynucleotide-terminated sequence and are displayed in Fig. 1.

the brain promoter may map to the 140-kb *Sfi* I fragment immediately upstream of the 700-kb fragment. This location was confirmed by the observation that both the muscle promoter (pERT84-10) and the brain promoter probes (pC + pD) hybridized to the same 840-kb partial digestion product (Fig. 4 *A* and *B*). To localize the brain promoter within the 140-kb *Sfi* I fragment, *Sal* I and *Sal* I/*Sfi* I digests were performed, since phage mapping (data not shown) revealed a *Sal* I site located 1 kb downstream of the transcription start site. Probe pD, on the 3' side of this *Sal* I site, recognized a 55-kb *Sal* I fragment that was reduced to 45 kb by *Sfi* I digestion (Fig. 4*D*), indicating that the brain promoter is 46 kb 5' to the *Sfi* I site. The muscle promoter probe (p84) hybridizes to a 135-kb *Sal* I fragment and an extensive series of partial digestion products (Fig. 4*B*) in common with the brain promoter, indicating that the brain and muscle promoters are located on adjacent *Sal* I fragments. To localize further the muscle promoter, we constructed a more refined restriction map using *Sal* I and *Sfi* I in combination with the enzymes *Xho* I, *Bam*HI, *Bgl* I, and *Kpn* I (data not shown). None of these digestions allowed precise localization of p84 relative to the *Sal* I sites, but together with information from mapping genomic phage clones of the region, we were able to estimate that the muscle promoter is at least 35 kb downstream of one *Sal* I site and 45 kb upstream of the other, giving a region of uncertainty of 55 kb. Thus, the muscle promoter is between 90 and 145 kb downstream from the brain promoter.

To link the promoters with the rest of the dystrophin gene, we hybridized the pulsed-field gel electrophoresis filters with a probe specific for exon two of the dystrophin gene, pD38 (a gift from R. Worton, Hospital for Sick Children, Toronto). This probe hybridized to a 390-kb *Sfi* I fragment in 6697 and GM1202 cells as well as a 700-kb fragment in all cell lines (data not shown). Analysis of double digests revealed that exon 2 is between 28 and 45 kb downstream of the partially digestable *Sfi* I site at 370 kb on our map (Fig. 5). This allowed placement of exon 2 relative to the promoters and exons 3–11, which have already been mapped relative to the *Sal* I and *Sfi* I sites at 600 and 800 kb, respectively, in Fig. 5 (19, 34–36). Thus, during transcription of the dystrophin gene in brain, the first splicing event removes a 405-kb intron, while transcription from the downstream muscle promoter requires excision of 240–310 kb of intron sequences, as diagrammed in Fig. 5.

Analysis of Promoter Mutations in Patients with Neuromuscular Disease. The observation that the dual promoters of

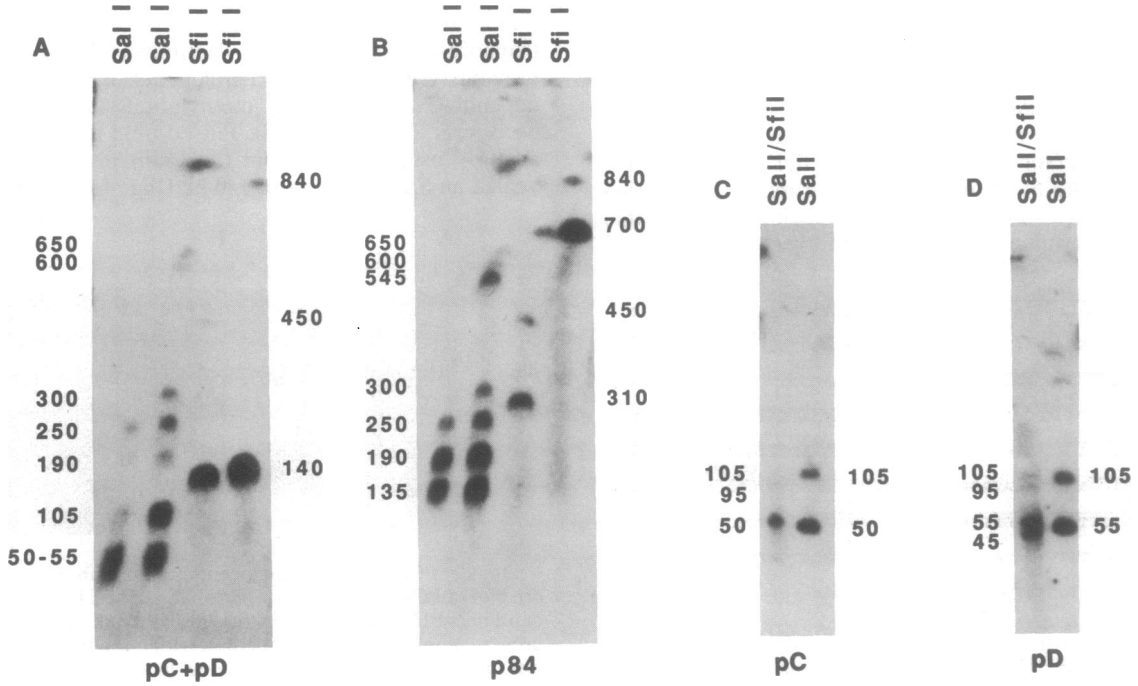

FIG. 4. Pulsed-field gel analysis of the dystrophin gene promoters. Cell blocks containing human DNAs were digested with *Sal* I, *Sfi* I, or both as indicated at the top of the figure; the probes used are indicated below the figure. Probes pC and pD are just upstream and just downstream, respectively, of the *Sal* I site at the brain promoter. Probe p84 contains the muscle promoter at the DXS142 locus. Sizes of relevant restriction fragments are indicated in kilobases and calculated from yeast and λ chromosome standards. Cell lines used were 6697 lymphoblasts (lanes 1 and 3 of *A* and *B*), GM5912 lymphoblasts (lanes 2 and 4 of *A* and *B*), and GM1202 lymphoblasts (*C* and *D*).

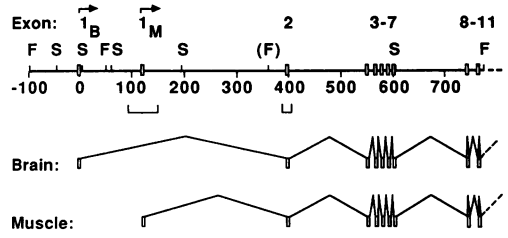

FIG. 5. Long-range restriction map of the 5′ end of the dystrophin gene. The two promoters (arrows) and exons 2–11 (vertical boxes) are mapped relative to *Sal* I (S) and *Sfi* I (F) sites in the first 800 kb of the dystrophin gene. The *Sfi* I site that is not normally cleavable in lymphocyte DNA is indicated by parentheses. The numerical scale (below) is in kilobases, and regions of uncertainty in placement of the muscle promoter and exon two are indicated by brackets under the scale. The predominant splicing patterns in brain and muscle are schematically illustrated at the bottom. Data on placement of exons 3–11 are derived from previous studies (34–36).

dystrophin are separated by a great distance, taken together with the fact that the majority of DMD mutations are due to intragenic deletions of the dystrophin gene (37–39), suggests that some individuals may have loss of one of the dystrophin promoters while maintaining use of the other. Since deletion of the upstream promoter with retention of the downstream promoter would be predicted to result in individuals with severely reduced levels of dystrophin in the brain, but not in muscle, we examined >50 male patients with developmental delay or mental retardation, rather than muscle weakness, for the presence of each promoter. In these preliminary experiments, we have yet to identify a patient with deletion of the upstream "brain" promoter only (Fig. 6*A*).

Conversely, deletion of the downstream muscle promoter with retention of the remainder of the gene might be expected

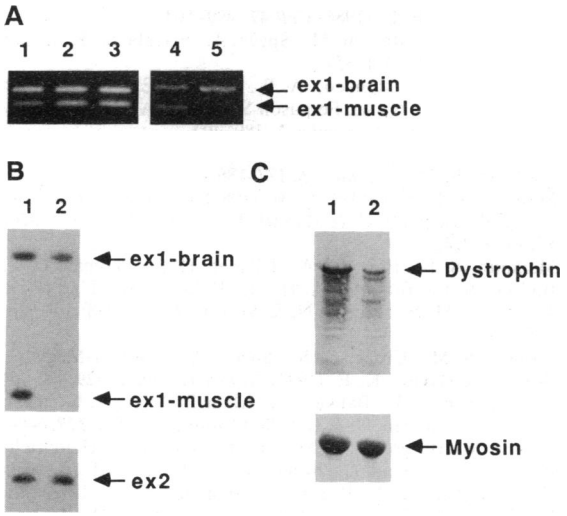

FIG. 6. Analysis of patients for dystrophin promoter mutations. (*A*) PCR analysis of DNA from male patients with mental retardation (lanes 1–3), Becker muscular dystrophy (lane 5) or a normal control (lane 4). The PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining. The bands corresponding to amplification of the brain and muscle promoters are indicated; no amplification of the muscle promoter is observed in patient 5. (*B*) Southern blot analysis of DNA from the Becker muscular dystrophy patient 5 (lane 5) above (lane 2) and from a normal control (lane 1). The expected positions of the first three exons of the DMD gene are indicated, confirming specific deletion of the muscle promoter in this patient. (*C*) Western blot analysis of muscle biopsy from the Becker muscular dystrophy patient (lane 2) and from a normal control (lane 1) using anti-dystrophin 60-kDa antibody (1). Shown at the bottom is Coomassie staining of the gel after transfer to allow normalization of the dystrophin staining relative to myosin. ex1, exon 1; ex2, exon 2.

to yield patients with decreased abundance of normal-sized dystrophin in muscle. We have previously identified several such patients with reduced amounts of normal-sized dystrophin (11). To determine if this was a result of deletion or rearrangement of either promoter, we performed PCR analysis on DNA from three male patients by using primers for each promoter. In one patient with reduced levels of full-sized dystrophin (Fig. 6*C*), the downstream "muscle" promoter could not be amplified from peripheral blood leukocyte DNA (Fig. 6*A*). Southern blot analysis using probes that recognize each promoter, as well as a probe for exon 2, confirmed that this patient had a deletion of the downstream promoter while retaining the flanking brain promoter and exon 2 (Fig. 6*B*). PCR analysis of DNA obtained from the muscle biopsy also did not detect the muscle promoter, making it unlikely that the reduced levels of dystrophin were due to somatic mosaicism in this individual. This 10-year-old patient has minimal muscle weakness consistent with mild Becker muscular dystrophy and is intellectually normal. Although it is possible that deletion of the muscle promoter may lead to different phenotypes in other individuals, we have not found specific deletion of the muscle promoter in any other case of DMD, Becker muscular dystrophy, or normal controls.

DISCUSSION

Dystrophin transcripts in brain contain an alternative 5′ terminal sequence from that found in muscle (16, 17). We have isolated the genomic region encoding this alternative exon and have mapped this exon >90 kb upstream of the previously described muscle promoter. Thus, this exon cannot result from alternative splicing from the muscle promoter and must be transcribed from an alternative upstream promoter. We have mapped the 5′ terminus of this alternative exon to within ≈30 bp by using PCR on human brain RNA. Using primer extension analysis on a transfected construct from this region, we have located two major transcriptional start sites within this 30 bp region and two minor start sites slightly further upstream. Although we cannot rule out that these sites are due to premature truncation by reverse transcriptase or artifactual initiation in the transfected construct, the close correspondence of the observed start sites with the PCR mapping of the endogenous transcripts suggest that the endogenous gene initiates transcription at these sites.

The magnitude of the spacing between promoters has several implications. The distance between the brain promoter and exon 2 represents the largest intron yet described (400 kb) and extends the total size of the gene to 2.4 million base pairs (40). The upstream promoter region described here represents the extreme 5′ terminus of the gene and may now be used to find polymorphic 5′ flanking markers for linkage studies of the DMD gene. Linkage analysis is necessary for carrier detection and prenatal diagnosis in the one-third of DMD mutations that do not show detectable deletions of the DMD gene. The large distance between the promoters allows for one promoter to be disrupted without affecting the other in some individuals. In particular, it is expected that deletions may extend from far upstream of the DMD gene, a region that may be asymptomatically deleted, to breakpoints between the two promoters. Such deletions would result in individuals with normal muscle dystrophin that lack neuronal dystrophin. Due to the association of mental retardation with DMD, we predict that such deletions may be among the heterogeneous causes of X chromosome-linked mental retardation (reviewed in ref. 41). A preliminary search for such patients has not yet been fruitful. However, like the mental retardation associated with DMD, it is expected that only one-third of such individuals with this deletion would be considered mentally retarded. It is also possible that the low level

expression of the muscle promoter in the brain (16, 18) is sufficient for normal brain function in the absence of the brain promoter. Finally, it is interesting to note that one study of X chromosome-linked mental retardation has found suggestive evidence of linkage to the DMD gene (42); the use of the PCR analysis described here should resolve whether the mental retardation found in such families is due to deletion of the dystrophin brain promoter.

We have identified a patient with low levels of dystrophin in muscle and specific deletion of the downstream muscle promoter. Dystrophin transcription in this patient must therefore occur from an alternative promoter. Low levels of the brain transcript have been previously described in muscle cells (16, 18). Thus, the dual promoters of dystrophin do not show absolute tissue specificity as their names currently in usage imply, and the use of more systematic names (P1 and P2) might be preferable. In any case, this low amount of the brain-type transcript might be sufficient to produce the reduced level of dystrophin observed in this patient. Alternatively, it is possible that a cryptic promoter is activated in this patient. Discrimination between these possibilities would require additional amounts of muscle biopsy material beyond that which we obtain for Western blot analysis. Nevertheless, it is hoped that the use of the probes described here for the upstream promoter may lead to better analyses in other patients with similar phenotypes.

Analysis of the promoter structure of the DMD gene has led to the prediction of individuals with tissue-specific promoter disruptions. In return, analysis of these patients may lead to insight into the function of this cytoskeletal gene. Such patients may be particularly critical for understanding the role of dystrophin in the brain, since analysis of the cognitive deficits of DMD patients may be complicated by the decreased motor function of these individuals. Thus, identification of individuals with specific disruption of the brain promoter, using the probes described here, may ultimately shed light on the function of dystrophin in the human brain.

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